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Effect of biochars pyrolyzed in N_2 and CO_2, and feedstock on microbial community in metal(loid)s contaminated soils

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ABSTRACT

Little is known about the effects of applying amendments on soil for immobilizing metal(loid)s on the soil microbial community. Alterations in the microbial community were examined after incubation of treated contaminated soils. One soil was contaminated with Pb and As, a second soil with Cd and Zn. Red pepper stalk (RPS) and biochars produced from RPS in either N_2 atmosphere (RPS_N) or CO_2 atmosphere (RPS_C) were applied at a rate of 2.5% to the two soils and incubated for 30 days. Bacterial communities of control and treated soils were characterized by sequencing 16S rRNA genes using the Illumina MiSeq sequencing. In both soils, bacterial richness increased in the amended soils, though somewhat differently between the treatments. Evenness values decreased significantly, and the final overall diversities were reduced. The neutralization of pH, reduced available concentrations of Pb or Cd, and supplementation of available carbon and surface area could be possible factors affecting the community changes. Biochar amendments caused the soil bacterial communities to become more similar than those in the not amended soils. The bacterial community structures at the phylum and genus levels showed that amendment addition might restore the normal bacterial community of soils, and cause soil bacterial communities in contaminated soils to normalize and stabilize.

1. Introduction

Soils are often contaminated with metal(loid)s due to anthropogenic activities such as mining, industrial processes, and agricultural practices (Li et al., 2014; Rizwan et al., 2016). High metal(loid) contamination levels in arable soils lead to contamination of the food chain via plant absorption, and to surface and ground water contamination (Ahmad et al., 2016b). Hence, scientists have examined potential cost-effective methods to reduce the bioavailability of metal(loid)s in arable soils, and thus allow for safe crop production on these soils (Mohamed et al., 2015; Rehman et al., 2017). Biochar has emerged as an efficient and sustainable amendment that can be used for the immobilization of soil metal(loid)s and concomitantly improves overall soil quality (Igalavithana et al., 2017b; Yang et al., 2019). Biochar has been found to cause significant reductions in the availability of metal(loid)s in contaminated soils due to the formation of stable metal(loid) compounds via a variety of mechanisms such as ion exchange, surface precipitation, surface complexation, electrostatic attraction, and organo-mineral complexation (Ahmad et al., 2014, 2012; Lu et al., 2016; Park et al., 2016). The ability of different biochars to immobilize metal(loid)s has been widely tested in contaminated soils with different metal(loid)s at different contamination levels, and under a variety of physicochemical conditions (Ahmad et al., 2016a; Igalavithana et al., 2017a; Yang et al., 2019). Both successful and unsuccessful results have been reported (Fang et al., 2016; Igalavithana et al., 2017b; Li et al., 2016; Van Poucke et al., 2017). The majority of existing studies have focused on the extent of metal(loid) immobilization and changes in soil chemical properties (Huang et al., 2019; Penido et al., 2019; Qian et al., 2019).

Recently, the impact of biochar on soil microbial communities has

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been studied (Ahmad et al., 2016c; Igalavithana et al., 2017a; Moore et al., 2018; Xu et al., 2017). Igalavithana et al. (2017b) examined the soil microbial communities after incubating contaminated soils for 30 days with biochars produced from crop residues (i.e., wood bark, cocopeat, and palm kernel shells) at 500 °C using fatty acid methyl ester (FAME) analysis. None of the specific microbial groups (i.e., Gram-positive bacteria, Gram-negative bacteria, unspecified bacteria, fungi, actinomycetes, and arbuscular mycorrhizal fungi) showed a significant increase after the incubation period, even though the exchangeable lead (Pb) concentration was reduced by biochar application. Lead, copper (Cu), and antimony (Sb) immobilization and microbial community alteration by soybean stover and pine needle biochars pyrolyzed at 300 and 700 °C were examined in a shooting range soil by Ahmad et al. (2016c); the two biochars produced at 300 °C significantly decreased Pb and Cu contents and increased total bacteria, Gram-negative bacteria, and actinomycetes in the soil. Bashir et al. (2018b) observed increased carbon, nitrogen, and microbial biomass in contaminated soil after cadmium (Cd) immobilization by rice straw, rice hull, and maize stover biochars. Zhou et al. (2018) examined microbial biomass, *nifH* gene abundance, and nitrogen transformations in the rhizosphere of wheat growing on a Pb- and Cd-contaminated soil after field application of a commercial biochar. Microbial biomass and nitrogen transformation increased and metal mobility significantly decreased when 40 $t \cdot ha^{-1}$ biochar was added to rhizosphere soil over two consecutive years. Moreover, *nifH* gene abundance in the rhizosphere increased in both years for both biochar application rates.

Nonetheless, detailed assessments of soil microbial communities are not performed at an adequate level in most studies, and hence there is a large research gap regarding biochar application and microbial community behavior in metal(loid)-contaminated soils (Blagodatskaya and Kuzyakov, 2013; Dangi et al., 2012; Igalavithana et al., 2017a; Yang et al., 2019). Soil microorganisms support all soil functions and keep the soil in a healthy condition (Lehmann et al., 2011). Generally, the beneficial microorganisms in metal(loid)-contaminated soils are depleted. Therefore, natural soil functions are reduced (Shaheen and Rinklebe, 2015). Successful metal(loid) immobilization methods must not only be evaluated in terms of efficiency and cost effectiveness, but also on their ability to enhance the soil microbial community (Mukhopadhyay et al., 2013). Soil microbial community is broad to study in detail in a single study. Therefore, this study was conducted to investigate the response of the soil bacterial community to the application of (1) red pepper stalk biomass, or (2) biochars produced from this feedstock in either N_2 or CO_2 atmosphere to metal(loid) contaminated soils. This is the first study that evaluates the effects of biochars produced under N_2 and CO_2 atmospheres on soil bacterial community. Biochars produced in CO_2 atmosphere typically exhibit a significantly higher surface area, aromaticity, pH and ash content than biochar produced in N_2 atmosphere (Lee et al., 2017). In a previous studies it was found that biochar produced in CO_2 reduced the exchangeable fraction of metal(loid)s and their kinetics of release significantly better than biochar conventionally produced in N_2 atmosphere (Igalavithana et al., 2018). Accordingly, it was hypothesized that biochar produced in CO_2 atmosphere would have stronger beneficial effects on the soil bacterial community than biochar produced in N_2 atmosphere. Amplicon sequencing technology, which sequences 16S rRNA genes using the Illumina MiSeq platform, was applied to analyze the bacterial community up to the genus level after a one-month incubation of two metal(loid)-contaminated soils under laboratory conditions.

2. Material and methods

2.1. Soil amendments

Red pepper stalk (RPS) (*Capsicum annuum* L.) and two biochars produced from RPS were used in this experiment as soil amendments. In brief, the biochars were prepared at 650 °C with a purging gas (either N_2 or CO_2) flow rate of 500 $mL \cdot min^{-1}$. The pyrolysis temperature was selected based on a preliminary study of soil metal(loid) immobilization (unpublished data). The biochars prepared with N_2 and CO_2 are subsequently referred to as RPS_N and RPS_C, respectively. Details of production conditions and the properties of the biochars can be found in Lee et al. (2017) and Igalavithana et al. (2017a). The biochars and RPS were ground and sieved through a 2-mm sieve prior to their use in the soil incubation study.

2.2. Soil properties and incubation study

An incubation experiment was carried out to assess the effects of the RPS and the two biochars on the microbial communities in contaminated soils. The metal(loid) contaminated soils used in the incubation study were collected from Korea (sandy loam, referred to herein as K-soil) (36°44′05″N, 127°12′12″E) and Belgium (sand, referred to herein as B-soil) (51°12′41″N; 5°14′32″E), and prepared by air drying and sieving through a 2-mm sieve. The K-soil was highly contaminated with Pb and As, whereas the B-soil was contaminated with Cd and Zn; both exceeded the soil contamination warning limits for Korea and Belgium, respectively (Table 1) (Igalavithana et al., 2017a). Previous mining activities near the K-soil sampling location are the likely source of Pb and As, whereas industrial activities conducted at the B-soil sampling site are responsible for the Cd and Zn contamination in B-soil (Igalavithana et al., 2018). The comparatively higher organic carbon percentage in K-soil (i.e., 5.76%) than in B-soil (i.e., 2.38%) might be due to the accumulation of plant-derived organic matter from

Table 1
Physicochemical properties of the studied soils (Igalavithana et al., 2017a).

Soil	Land use	Sand	Silt	Clay	Soil texture	OC[a]	pH[b]	EC[b,c]	Total exchangeable cations (ΣCa, K, Mg, Na)	Total			
										As	Pb	Cd	Zn
		%	%	%		%		dS m^{-1}	$cmol_{(+)}\ kg^{-1}$	$mg\ kg^{-1}$	$mg\ kg^{-1}$	$mg\ kg^{-1}$	$mg\ kg^{-1}$
K-soil[d]	Fallowed upland agriculture	79.9	9.2	10.8	Sandy loam	5.8	5.1	0.12	2.8	1940	1445	–	–
B-soil[e]	Fallowed upland agriculture	90.9	3.2	5.9	Sand	2.4	6.2	0.09	3.5	–	–	9.4	505
Soil contamination warning limit Korea (Ministry of Environment Korea, 2016)										25	200	4	300
Soil contamination warning limit, Belgium (Vlaamse Regering, 2008)										35	120	1.2	200

[a]OC, organic carbon.
[b]1:5 ratio of soil to deionized water.
[c]EC, electrical conductivity.
[d]Adjacent to Tancheon mine, Gongju-si, Chungcheongnam-do, Korea.
[e]Adjacent to an industrial area, Lommel, Limburg, Belgium.

past agricultural activities. Moreover, the high clay content in K-soil (i.e., 10.84%) might have contributed to preservation of organic matter as organo-clay complexes (Riefer et al., 2017). Both soils had slightly acidic pH values and a comparable amount of exchangeable cations.

For each combination of soil (i.e., K- or B-soil) and amendment (i.e., RPS, RPS_N, or RPS_C), 200 g soil and 2.5% (mass basis) of amendment were mixed well and introduced into a 600-mL high-density polyethylene bottle. Control treatments included the soil without amendments. A 0.45 μm syringe filter was affixed to the lid of each bottle to maintain aerobic conditions inside the bottles during the incubation period. The bottles were incubated at 25 °C in the dark for 30 days in an incubator (MIR-554, SANYO Electronic, Co., Ltd., Tokyo, Japan), and a water holding capacity of 70% was maintained in the soils over this period. Four replicates were maintained for each treatment. After 30 days, the soils were collected and aliquots of each were stored at −20 °C for microbial analysis. The remaining soils were air dried for analysis of chemical properties.

2.3. Soil bacterial properties

The total DNA of the soil bacterial community was extracted using PowerSoil DNA Isolation Kits (MO BIO Laboratories, Inc., USA), following the manufacturer's instructions. Each sequenced DNA sample was prepared according to the Illumina 16S Metagenomic Sequencing Library protocols to amplify the V3 and V4 regions (519F-816R) of the 16S rRNA genes. The DNA quality was measured using PicoGreen (Molecular Probes, USA) and Nanodrop (Thermo Scientific, USA). Genomic DNA (10 ng) was amplified by PCR. The barcoded fusion primer sequences used for amplifications were as follows: 519F: 5′ CCTACGGGNGGCWGCAG 3′; and 806R: 5′ GACTACHVGGGTATCTAATCC 3′. The final purified product was then quantified using qPCR according to the qPCR Quantification Protocol Guide (KAPA Library Quantification Kits for Illumina sequencing platforms) and qualified using the LabChip GX HT DNA High Sensitivity Kit (PerkinElmer, USA). The paired-end (2 × 300 bp) sequencing was performed by the Macrogen with the MiSeq™ platform (Illumina, USA). All samples were replicated there times.

After short and long reads were filtered, chimeric reads were identified and discarded using QIIME (Caporaso et al., 2010). The readings with more than 97% identity were clustered into an operational taxonomic unit (OTU) to calculate richness at the species level using CD-HIT (Fu et al., 2012). The representative sequence in each OTU was used to determine their taxonomic positions using UCLUST (Venkatavara Prasad et al., 2015) against the RDP 16S rRNA gene database (Maidak, 2001). Subsamples were obtained from the samples to obtain 18,000 sequences in each sample; this was done to reduce bias from different sample sizes. Calculation of observed OTUs, Chao1, Shannon, and Simpson's evenness indexes, and principal coordinate analysis (PCoA) of the weighted Unifrac matrix were performed with the subsamples using QIIME (Caporaso et al., 2010). Hierarchical heat maps of the bacterial community at the phylum, class, order, family, and genus levels were generated using R packages (R Core Team, 2018).

2.4. Phospholipid-derived fatty acid (PLFA) extraction and analysis

Dried soil (5 g) was placed into a thimble filter (ADVANTEC, Japan) and loaded into a Soxhlet extractor apparatus (PYREX, USA). $CHCl_3$:MeOH was used as an extraction solvent; 200 mL of CHCl3:MeOH 1:2 (v/v) was placed in a distillation flask and PLFA extraction was carried out at 100 °C for 6 h using a Soxhlet heating mantle (M TOPS, Korea). After the extraction process was completed, the solvent containing the extracts was evaporated under vacuum in an oven at 40 °C for 30 min.

Analysis of the extracted PLFA was conducted after pseudo-catalytic transesterification using silica gel (236799, Sigma-Aldrich, USA) as the porous media. A 0.25-inch bulkhead unit (SS-400-61, Swagelok, USA) was used as a batch-type reactor for confining the reactant and silica gel. One side of bulk head was sealed with a stainless-steel cap (SS-400-P, Swagelok, USA), and the bulkhead was partially filled with 0.3 g of the silica gel porous media. Next, 10 ± 0.2 mg of extracted PLFA was put into the bulkhead along with 200 μL of high purity methanol (34860, Sigma-Aldrich, USA). Another 0.3 g of silica gel was added to the bulkhead and it was sealed with another stainless-steel cap. The prepared bulkhead was placed in a box furnace (FX-05, DAIHAN Science, Korea) at a temperature of 400 °C for 10 min and then cooled to room temperature. This procedure converted PLFA into methyl ester, which was separated from the bulkhead and diluted with 3 mL of dichloromethane (439223, Sigma-Aldrich, USA). Finally, the sample was analyzed by GC (Agilent 7890B)/TOF-MS (Almsco Bench TOF-dx) coupled with a DB-Wax column, 30 m × 0.25 mm ID × 0.25 μm film (Agilent, USA). The injected volume was 1 μL. The GC oven conditions were programmed as follows: temperature was held at 50 °C for 10 min, then ramped from 50 to 240 °C at a rate of 10 °C·min^{-1}, and held at 240 °C for 50 min. The temperatures of the injector and detector were set to 240 °C. The ion source and transfer line temperatures of the TOF-MS were set to 200 and 250 °C, respectively. The filament emission and ionization currents were 1.7 A and 70 mV, respectively. Electron impact mass spectra were received in the 30–400 amu range at 1 s intervals. High purity (99.999%) gases (i.e., helium, air, and hydrogen) for operating the GC/TOF-MS were purchased from Green Gas in Korea. Three replicates were analyzed. Standards were analyzed after every ten samples to check the accuracy of the analysis.

2.5. Statistical analysis

Principal coordinate analysis of fatty acid profiles was performed using the Bray-Curtis dissimilarity matrix of the fatty acid abundance table using the scikit-bio python package (http://scikit-bio.org/). Pearson correlation analysis (SAS statistical software, ver. 9.3, Cary, NC, USA) was conducted between soil physicochemical properties and OTUs, Chao1, and Shannon indexes to determine the amendment effects on soil microbial richness and diversity. To examine the relationships between soil physicochemical properties and microbial species, a constrained ordination analysis (i.e., redundancy analysis (RDA) and canonical correlation analysis (CCA)) was conducted; and no specific trend could be identified.

3. Results and discussion

3.1. Soil bacterial richness

All rarefaction curves of observed OTUs were saturated and confirmed that the numbers of sequences sampled were high enough to reveal bacterial community diversity (Fig. S1). There were marked differences in the OTUs, Chao1, and Shannon indexes between the K-soil and the B-soil, with those of B-soil being much higher (Table 2). The rarefaction curves also showed that B-soil contained a higher number of bacterial species than K-soil. This indicates that the bacterial community in the B-soil is much more complex than that in the K-soil. The lower diversity in K-soil may be due to the more acidic condition (pH 5.1) than the B-soil (6.2) (Wu et al., 2017).

Changes in diversity indexes for the different amendment treatments showed less clear, but still noticeable, patterns. All soil amendments increased the Chao1 in both K-soil and B-soil, with the exception of a single sample (B-soil with RPS_N treatment). The increase of richness in the K-soil samples was more pronounced than in the B-soil, probably because they initially contained a smaller number of bacterial species, whereas many more bacterial species were initially present in the B-soil. The high pH of RPS_N and RPS_C (Table 3) made the overall pH of treated soil samples more neutral, which might also have contributed to the increased bacterial richness. Because the pH of K-soil was more acidic than that of B-soil, the neutralization effect on K-soil might be

Table 2
Bacterial diversity indexes and ammonium acetate extractable metal(loid)s (mg kg^{-1} dry soil) after incubation.

Soil	Treatment	Observed OTUs[d]	Chao1[d]	Shannon[d]	Simpson's evenness[d]	Pb[e] mg kg^{-1}	As[e] mg kg^{-1}	Cd[e] mg kg^{-1}	Zn[e] mg kg^{-1}	pH[e]
K-soil[a]	Control[c]	274	287	5.91	0.098	3.4	0.5	–	–	5.1
	RPS[c]	285	313	5.65	0.073	2.8	0.5	–	–	5.8
	RPS_N[c]	356	445	5.91	0.074	1.7	0.8	–	–	6.6
	RPS_C[c]	339	408	5.60	0.048	1.1	1.1	–	–	7.0
B-soil[b]	Control[c]	687	762	7.54	0.105	–	–	3.0	83.3	6.2
	RPS[c]	667	830	6.75	0.045	–	–	2.5	66.8	6.8
	RPS_N[c]	659	725	7.16	0.072	–	–	2.3	54.5	7.6
	RPS_C[c]	692	780	7.37	0.080	–	–	2.2	53.6	7.8

[a] Adjacent to Tancheon mine, Gongju-si, Chungcheongnam-do, Korea.
[b] Adjacent to an industrial area, Lommel, Limburg, Belgium.
[c] Control, RPS, RPS_N, and RPS_C represent the soils with no amendment, red pepper stalk amendment, and the two biochar amendments produced from red pepper stalk in N_2 or CO_2 atmosphere, respectively.
[d] All diversity indexes were calculated based on the subsamples adjusted to contain 18,000 sequences in each sample. Valid reads were in the range of 18,166–33,763, and the total was 205,414.
[e] Igalavithana et al. (2018).

more pronounced than on B-soil, even though the final pH of K-soils remained lower than that of the B-soils (Table 3). Neutral or slightly alkaline pH values are more favorable for soil bacterial growth (Rousk et al., 2009). The amendments have high surface areas, have a neutralization effect, and provide additional nutrients, all of which may have contributed to the observed increase in microbial richness (Lehmann et al., 2011). Igalavithana et al. (2017a) observed a significantly high bacteria population in soils treated with biochar characterized by a high percentage of mobile matter due to the supplement of readily available carbons. Moreover, a considerably high ash content favours metal(loid) immobilization, and thus may have increased the bacterial growth as a result of the reduction of metal(loid) toxicity and the supply of essential minerals (Ahmad et al., 2016a; Ippolito et al., 2012; Kołodyńska et al., 2012).

The addition of RPS_N and RPS_C may have supplied an additional surface for bacterial attachment and growth (Lehmann et al., 2011). RPS_C contributed the largest additional surface area (Table 3), which may have greatly increased bacterial attachment and proliferation in K-soil. However, apparently the addition of readily available carbon sources, rather than surface area, was more important in favoring microbial growth in the B-soil; this might be attributable to the initial low soil organic carbon content (i.e., 2.38%) in that soil (Blagodatskaya and Kuzyakov, 2013), further studies are needed for the confirmation. The large decrease of Pb in the K-soil (i.e., 67%) after RPS_C addition (Table 3) might also have had a positive influence on bacterial richness (Ahmad et al., 2016d; Bashir et al., 2018a; Igalavithana et al., 2017a). In addition, the increase in ammonium acetate extractable As due to the soil amendments apparently did not adversely impact bacterial richness in the K-soil. This observation agrees with Igalavithana et al. (2017a). This might reflect tolerance to As of the prevailing bacterial community due to long-term exposure (Lorenz et al., 2006). The reductions in Cd (i.e., RPS_N 25%, RPS_C 27%) and Zn (i.e., RPS_N 35%, RPS_C 36%) after biochar addition in the B-soil did not have a significant impact on bacterial richness. Similarly, Zhu et al. (2013) observed no linear relationships between soil metal(loid) contents and bacterial richness. The authors explained that metals do not simply affect a single species, but the whole microbial population. Hence, the formation of metal (loid) adaptive species and the disappearance of vulnerable species during long-term contamination maintain the bacterial richness in a static state, even after reductions in metal(loid) caused stresses in the short-term (Xie et al., 2016).

3.2. Evenness and diversity of bacterial communities

Trends in bacterial diversity (Shannon index) were not comparable with those of bacterial richness (Table 2). The Shannon index decreased in all samples treated with amendments except for one (K-soil with RPS_N treatment), meaning that diversity decreased even though richness increased. The controls for both soils showed higher bacterial diversities than the amendment-treated soils. Hence, it was apparent that soil amendments increased the growth of certain bacterial species in contaminated soils. This may be due to the differences in growth factors among bacterial species (Blagodatskaya and Kuzyakov, 2013). This phenomenon can be clearly explained in terms of evenness changes; evenness values were significantly reduced after amendment treatments (Table 2). The supplementation of carbon and nutrients from the amendments possibly provided beneficial conditions for specific bacterial strains in the B-soil. As these strains outgrew other strains, the evenness was decreased. The increase in richness and decrease in evenness had counteracting effects on the Shannon index values as both values are directly involved in the calculation of the Shannon index, but the decrease in evenness had a greater impact in this case (Southwood and Henderson, 2000). Moreover, the decrease in available metal(loid)s in B-soil might have positively impacted the growth of some suppressed species in the community, causing them to outgrow others due to the favorable environmental conditions. Zhang et al. (2016) also observed higher microbial community diversity in slightly metal(loid)-contaminated soils than in highly contaminated soils, and reported strong negative correlations between microbial biomass and Cr, Cd, and Pb levels. The highest bacterial diversity was observed in the RPS_N treatment for the K-soil, and in the RPS_C treatment for the B-soil. This shows that the reduction in metal(loid)s by biochars contributes to maintenance of the soil microbial diversity in contaminated soils. As biochar provides additional growth surfaces and essential nutrients, a high soil bacterial diversity can be expected in biochar-amended soils (Steiner et al., 2004). Also, the increased pH will have contributed to the increase in bacterial diversity (Maier and Pepper, 2009; Rousk et al., 2009). Fierer and Jackson (2006) reported a strong correlation between soil pH and soil bacteria and observed significant alterations in bacterial community with respect to the variation of soil pH. Similarly, Lauber et al. (2009) observed a strong correlation among soil bacterial community and pH, and close to natural pH values were identified as optimal for high bacterial diversity. In this study, Chao1 and OTUs were positively correlated with pH in the K-soil, reflecting that the microbial richness increased with an increase in soil pH following application of the amendments. However, soil pH was not correlated with the Shannon index in either soil. Other soil properties were not correlated with the Chao1, OTUs, or Shannon indexes (data not shown).

The B-soil with a sandy texture unexpectedly showed a higher microbial diversity than did in K-soil. This is more likely to be caused by the relatively low metal contents in B-soil (i.e., 9.4 mg·kg^{-1} Cd and 505 mg·kg^{-1} Zn) compared to K-soil (i.e., 1940 mg·kg^{-1} Pb and

Table 3
Physicochemical characterization of the feedstock and biochar (Lee et al., 2017).

Amendment	Pyrolysis temperature	Purged gas	Moisture	Mobile matter	Fixed C	Ash	C[b]	H[b]	N[b]	O[b]	H/C	O/C	pH[c]	EC[c]	Surface area[d]	APV[d,f]	APD[e,g]
	°C		%	%	%	%	%	%	%	%				dS m^{-1}	m^2 g^{-1}	$\times 10^{-3}$ m^3 g^{-1}	nm
RPS[a]	–	–	4.34	82.50	11.25	1.91	49.78	6.46	1.09	42.67	1.56	0.64	6.02	0.06	NA[h]	NA[h]	NA[h]
RPS_N[a]	650	N_2	2.52	22.04	66.14	9.30	86.63	2.42	2.62	8.34	0.33	0.07	12.19	0.20	32.46	0.02	3.79
RPS_C[a]	650	CO_2	3.33	22.71	62.05	11.91	83.85	2.22	2.56	11.36	0.32	0.10	9.50	0.11	109.15	0.09	2.64

[a] RPS, RPS_N, and RPS_C represent the red pepper stalk and the biochars produced from red pepper stalk in N_2 or CO_2 atmosphere, respectively.
[b] Moisture- and ash-free.
[c] 1:20 ratio of biochar to deionized water.
[d] APV is the average pore volume.
[e] APD is the average pore diameter.
[f] Brunauer-Emmett-Teller (BTE) method.
[g] Barret-Joyner-Halender (BJH) method.
[h] NA means not analyzed.

1450 mg·kg^{-1} As) and a favorable soil pH for bacterial growth. The high ash contents and surface areas of the two biochars might also have had a positive impact on the microbial diversity in the two soils, which was greater than that in the RPS treatment (Igalavithana et al., 2017a, 2017b). In addition, the two biochars showed an enhanced ability to immobilize soil metal(loid)s and had higher stability than RPS; thus, incorporation of the biochars into metal(loid)-contaminated K-soil can be expected to maintain an improved microbial diversity over the longer term. There was no substantial difference between the two biochars with respect to the increase in bacterial richness and diversity in soils during this short-term laboratory incubation. However, the significantly high surface area and ash content of RPS_C can be expected to enhance the soil microbial richness and diversity compared to RPS_N in the long term (Lehmann et al., 2011).

3.3. PCoA analysis of weighted Unifrac matrix

The first two principal coordinates of the principal coordinate analysis (PCoA) of OTUs explained 48.27% and 26.90% of the variability, respectively (Fig. 1a). The two soils were clearly separated into two groups, with the exception of the K-soil control, which highly deviated from the other soils. The PCoA analysis confirmed that the application of amendments to the K-soil significantly altered the bacterial community and its diversity. Interestingly, the K-soil and B-soil became more similar after amendment treatments; the distances between the samples in the coordinate space were reduced after treatments. This implies that the amendment treatments may have caused the bacterial communities of the different soils to converge, even though the initial bacterial communities were quite different. This might be due to the increased pH in the K-soil which favored the growth of bacteria (Rousk et al., 2009). Cluster analysis showed the separation of soils into apparent clusters (Fig. 1b); it also confirmed that the K-soil control deviated significantly from all other samples. In addition, of all the samples, the RPS_N and RPS_C treatments were consistently most closely connected in both soils. The effects of the two biochars on the bacterial communities were more similar than those of RPS. This may be due to the approximately similar chemical composition (mobile matter %, fixed C %, ash %, C %, H % and N %) of the two biochars. Igalavithana et al. (2017b) also did not observe differences in microbial communities in metal(loid) contaminated soils treated with biochars of comparable chemical compositions, produced at 500 °C from three crop residues (i.e., wood bark, cocopeat, and palm kernel shell). Both PCoA and cluster analysis showed that all three soil amendments had a greater influence on the bacterial diversity of the K-soil than of the B-soil; this is likely because the B-soil initially had a greater bacterial diversity than the K-soil, as observed in the controls. This might be due to the enhanced priming effect in the K-soil compared to the B-soil due to the increase in pH of the K-soil to more favorable conditions for microbial development (Hamer et al., 2004; Kuzyakov et al., 2009; Lehmann et al., 2011). The pH of the non-amended B-soil was at 6.2 which initially was already more favorable than that of the K-soil (i.e., 5.1) as explained in Section 3.2. Soil physicochemical factors, especially the soil texture of the K-soil (i.e., sandy loam), can also have had a positive influence on the growth of microorganisms and therefore, the bacterial diversity in K-soil may have increased responding to the increased pH (Kuzyakov and Blagodatskaya, 2015). It was predicted, based on the results of the one-month incubation experiment, that microbial richness in the K-soil will be more stable in the long run than that in the B-soil.

3.4. Soil bacterial community structure at the phylum level

On average, 11 phyla showed an abundance of more than 1%: (in descending order) Proteobacteria; Actinobacteria; Verrucomicrobia; Bacteroidetes; Chloroflexi; Planctomycetes; Firmicutes; Acidobacteria; candidate division WPS-1; Gemmatimonadetes; and Candidatus Saccharibacteria (Fig. 2). According to the meta-analysis conducted by

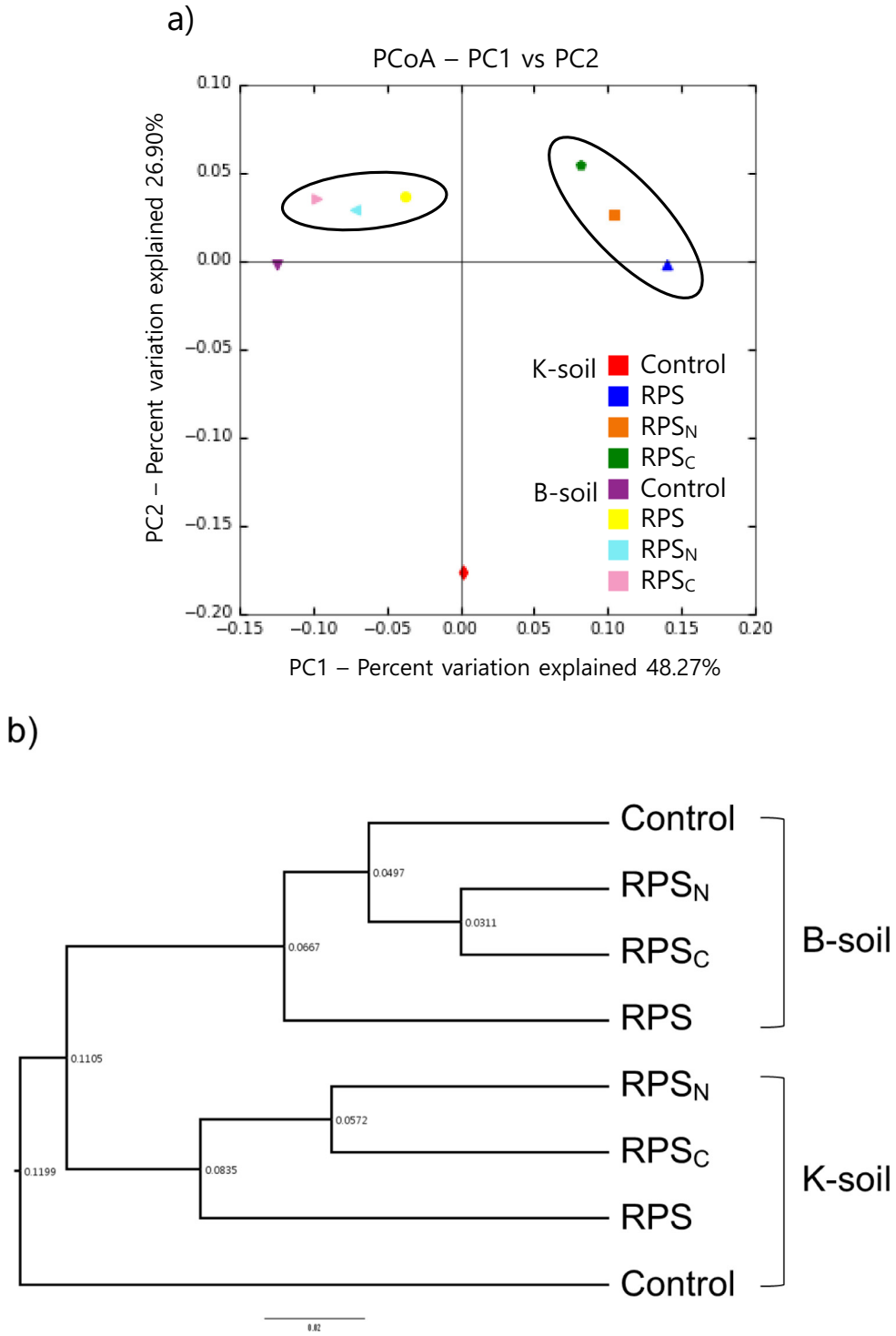

Fig. 1. a) Principal coordinate analysis (PCoA) and b) cluster analysis (UPGMA tree) based on the weighted Unifrac matrix. K-soil and B-soil represent the Korean soil and Belgium soil, respectively. Control, RPS, RPS_N, and RPS_C represent the soils with no amendment, red pepper stalk amendment, and the two biochar amendments produced from red pepper stalk in N_2 or CO_2 atmosphere, respectively.

Janssen (2006), the most common and abundant bacteria phyla in a vast range of forest soils are Proteobacteria, Acidobacteria, Actinobacteria, Verrucomicrobia, Bacteroidetes, Chloroflexi, Planctomycetes, Gemmatimonadetes, and Firmicutes, in descending order. All of these phyla were observed in all soil samples, and the order of abundance was also identical for the first six phyla, with the exception of Acidobacteria.

Soil alterations due to metal(loid) contamination may have changed the natural abundance of bacterial phyla in soils (Burges et al., 2015; He et al., 2017). After amendment, microbial communities in both soils appear to become more similar to each other, and might regain their natural diversity in terms of phyla compositions. Proteobacteria was the most abundant phylum in all samples except for the control of B-soil. Its abundance decreased with the addition of amendments in the K-soil, but increased after amendment addition in the B-soil, where it became the most abundant phylum, as in the other treatments. According to Spain et al. (2009), Proteobacteria include significant morphological,

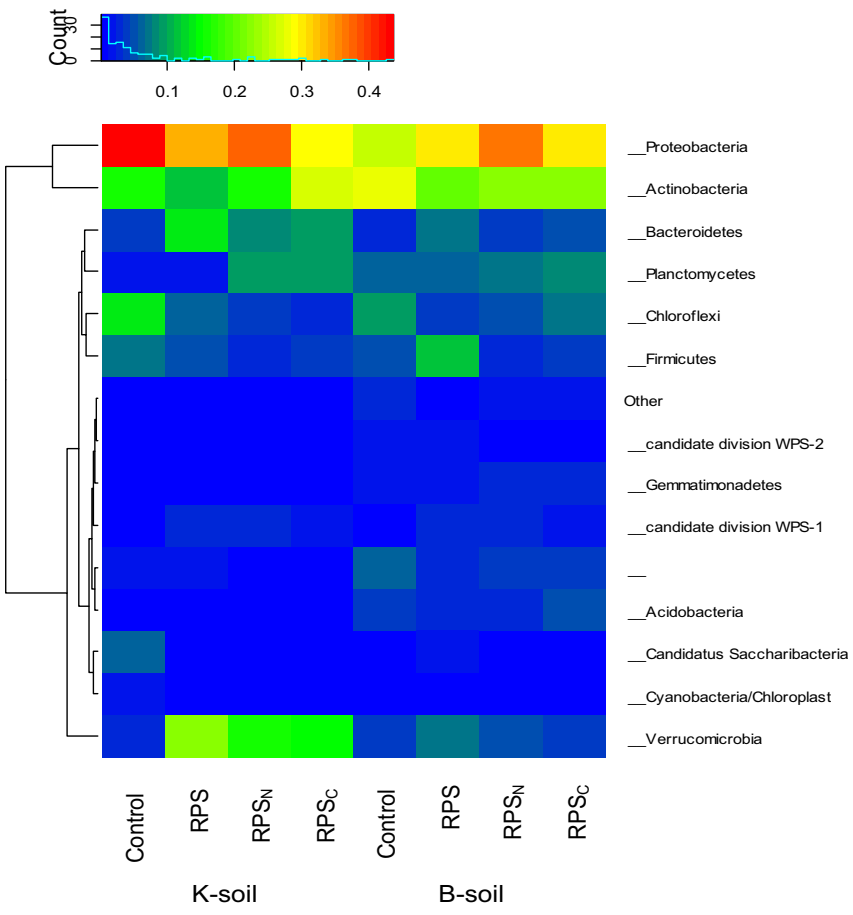

Fig. 2. Heat map of absolute abundance of bacterial phyla in soils after the incubation period. K-soil and B-soil represent the Korean soil and Belgium soil, respectively. Control, RPS, RPS_N, and RPS_C represent the soils with no amendment, red pepper stalk amendment, and the two biochar amendments produced from red pepper stalk in N_2 or CO_2 atmosphere, respectively.

physiological and metabolic diversity. They are vital in carbon, nitrogen and sulfur cycling. Amendments might have provided the required growth conditions for Proteobacteria by altering soil pH and supplying readily available nutrients. Moreover, this phylum is considered as one of the large bacteria phyla. Thus, the growth of a large number of species may reflect a great abundance of Proteobacteria after the incubation period (Miyashita, 2015). The most pronounced change was the increase in abundance of Verrucomicrobia; it initially constituted only a small proportion of the total organisms (i.e., 2.5% and 3.3% in K-soil and B-soil, respectively), but increased by 5.8- to 8.7-fold in the K-soil and 1.2- to 2.0-fold in the B-soil after amendment treatments. Bacteroidetes also increased after addition of amendments in both the K-soil (i.e., 2.0–3.3-fold) and the B-soil (i.e., 1.3–2.6-fold). On the contrary, the initial relatively high proportions of Chloroflexi (i.e., 13.9% and 9.5% in K-soil and B-soil, respectively) decreased in the K-soil (to 2.9–6.0%) and in the B-soil (to 3.6–6.6%) after treated with amendments. For the soils treated by biochars, RPS_N and RPS_C, there was a noticeable increase in Planctomycetes in the K-soil (8.2–8.5-fold) and the B-soil (1.3–1.4-fold); this was different than for the RPS treatment (1.5- and 1.0-fold for K-soil and B-soil, respectively), implying that the changes in Planctomycetes are related to the biochars. The reduced proportions of Firmicutes also appear to be related to the biochars. In addition, the changes in abundance were similar between the biochar treatments for Verrucomicrobia and Bacteroidetes in both the K- and B-soil. According to previous studies Verrucomicrobia and Bacteroidetes abundance could be increased with the availability of carbon sources in soil, which in this study were applied through the amendments (Bergmann et al., 2011; Wolińska et al., 2017).

The two most abundant phyla observed in this study, Proteobacteria and Actinobacteria, are also generally the most abundant in soils around the world (Faoro et al., 2010). Proteobacteria is a key phylum that includes the Gram-negative bacteria that live symbiotically in plant roots. These bacteria carry out specific functions in soils, including biological nitrogen fixation, and oxidation of iron and methane (Itävaara et al., 2016). Actinobacteria are exploited as bioinoculants in agriculture due to their effect in enhancing plant growth and yield via several mechanisms, including biological nitrogen fixation; P, K, and Zn solubilization; production of plant growth hormones; and production of antagonistic substances (Yadav et al., 2018). Verrucomicrobia, which increased after treatment with amendments in this study, are ubiquitous in soils; in one study, they were detected in 180 out of 181 sites, with an average proportion of 23% (Wolińska et al., 2017). Bacteroidetes are widespread in many distinct habitats and soils in temperate, tropical, and polar ecosystems. The reduction in OTU number for Bacteroidetes was suggested as an indicator of agricultural usage (Wolińska et al., 2017). More specific studies are required to further confirm the reasons for the differences in bacteria phyla.

3.5. Soil bacterial community structure at the genus level

The bacterial community structure has been reviewed at the genus level in detail because even in the same phylum, the roles of different genera in the environment can be completely different. The B-soil revealed a much higher diversity in bacterial genus than the K-soil, but the effect of RPS and biochars on genus number was more intense for the K-soil (Table 4). The richness of genera was even reduced in the RPS and RPS_N treatments (i.e., −2% and −7%, respectively) of the B-soil. The differences in genus and species richness in the treated soils may be due to the higher biological toxicity of Pb, present in the K-soil, compared to Cd and Zn in the B-soil (Gu et al., 2017; Khan et al., 2010). Thus, the application of RPS and biochars might have had a greater effect in improving soil carbon and nitrogen cycles and the conversion

Table 4
Abundance (%) differences for each treatment, with most abundance taxa at the genus level.

Taxon	K-soil[a]				B-soil[b]			
	Control[c]	RPS[c]	RPS_N[c]	RPS_C[c]	Control[c]	RPS[c]	RPS_N[c]	RPS_C[c]
Total number of genera (at least one occurrence)	115	132	147	140	185	183	177	188
Actinobacteria; *Actinobacteria*; *Actinomycetales*; *Nocardiaceae*; *Nocardia*	3.7	0.9	6.5	22.0	0.0	0.0	0.0	0.0
Verrucomicrobia; *Spartobacteria*; *Spartobacteria_genera_incertae_sedis*	2.3	20.8	14.9	13.5	2.6	4.8	3.1	2.3
Proteobacteria; *Gammaproteobacteria*; *Xanthomonadales*; *Xanthomonadaceae*; *Rhodanobacter*	13.3	2.3	0.1	1.8	0.0	0.0	0.0	0.0
Chloroflexi; *Ktedonobacteria*; *Ktedonobacterales*	11.5	4.5	3.2	1.9	0.1	0.0	0.0	0.0
Bacteroidetes; *Sphingobacteriia*; *Sphingobacteriales*; *Chitinophagaceae*; *Niastella*	0.7	11.3	2.4	0.9	0.0	1.2	0.4	0.1
Firmicutes; *Bacilli*; *Bacillales*; *Bacillaceae 1*; *Bacillus*	4.7	2.2	2.4	2.6	2.0	10.9	2.4	1.1
Proteobacteria; *Betaproteobacteria*; *Burkholderiales*; *Oxalobacteraceae*; *Massilia*	4.2	7.9	9.3	4.8	7.0	10.7	7.9	4.1
Proteobacteria; *Alphaproteobacteria*; *Sphingomonadales*; *Sphingomonadaceae*; *Sphingomonas*	5.2	2.1	7.5	3.2	5.6	3.6	7.8	10.2
Actinobacteria; *Actinobacteria*; *Actinomycetales*; *Micrococcaceae*; *Arthrobacter*	4.5	3.0	4.8	2.8	6.9	10.1	7.4	7.8
Planctomycetes; *Planctomycetia*; *Planctomycetales*; *Planctomycetaceae*; *Pirellula*	0.0	0.7	8.4	9.1	1.2	2.3	3.1	2.8
Actinobacteria; *Actinobacteria*; *Gaiellales*; *Gaiellaceae*; *Gaiella*	0.4	0.4	0.4	0.2	6.9	1.6	3.5	3.4
Proteobacteria; *Betaproteobacteria*; *Burkholderiales*; *Burkholderiaceae*; *Burkholderia*	1.7	6.3	0.5	0.1	0.1	1.0	0.3	0.1
Candidatus Saccharibacteria; *Saccharibacteria_genera_incertae_sedis*	5.9	0.0	0.3	0.5	0.7	1.2	0.4	0.5
Proteobacteria; *Betaproteobacteria*; *Burkholderiales*; *Oxalobacteraceae*; *Noviherbaspirillum*	0.2	1.1	5.8	2.1	1.2	0.5	3.7	0.8
Proteobacteria; *Deltaproteobacteria*; *Myxococcales*; *Cystobacteraceae*; *Cystobacter*	0.0	0.1	0.3	2.4	2.2	2.4	4.9	4.3
Chloroflexi	1.0	0.6	0.6	0.6	4.2	2.1	2.4	2.8
Proteobacteria; *Betaproteobacteria*; *Nitrosomonadales*; *Nitrosomonadaceae*; *Nitrosospira*	3.8	0.2	1.0	0.4	1.4	0.0	0.6	1.0
Proteobacteria; *Gammaproteobacteria*; *Pseudomonadales*; *Pseudomonadaceae*; *Pseudomonas*	3.7	0.0	0.0	0.0	0.0	0.0	0.0	0.0

[a] Adjacent to Tancheon mine, Gongju-si, Chungcheongnam-do, Korea.
[b] Adjacent to an industrial area, Lommel, Limburg, Belgium.
[c] Control, RPS, RPS_N, and RPS_C represent the soils with no amendment, red pepper stalk amendment, and the two biochar amendments produced from red pepper stalk in N_2 or CO_2 atmosphere, respectively.

of toxic metal(loid) species to less toxic ones in the K-soil compared to the B-soil. Moreover, texture and organic carbon percentage of soils also may have influenced the resulted genus and species richness. The different response to amendment additions in terms of richness may be due to differences in initial richness of bacteria, neutralization effects, and soil properties (e.g., sandy vs. sandy loam soils).

The highest absolute abundances in bacterial genera were found in genera *Nocardia* and *Spartobacteria* genera *incertae sedis* in K-soil. Neither of these two genera was found in the B-soil. *Nocardia* was highest in RPS_C, followed by RPS_N, but it decreased compared to the control in the RPS treatment. *Nocardia* belongs to the phylum Actinobacteria. It is an important bacterial genus in agricultural soils because it is involved in demethylation, decarboxylation, and hydroxylation of phenolic compounds derived from lignin decomposition, and produces compounds for the formation of humus or dearomatization of aromatic compounds (Malarczyk et al., 1994). Moreover, *Nocardia* has been isolated from the rhizosphere of numerous crops, such as wheat, rice, sugarcane, pea, and maize (Yadav et al., 2018). The considerably high organic carbon content might be a reason for the presence of *Nocardia* in the K-soil. Moreover, based on the above results, it is apparent that RPS_C significantly increased soil biological functions and returned the soil back into a more healthy condition with respect to microbial activity. *Spartobacteria* genera *incertae sedis* was abundant in all amended K-soils, and in the RPS-treated B-soil. The class *Spartobacteria* was most dominant in the phylum Verrucomicrobia, accounting for 92% abundance in a survey of 181 soils (Wolińska et al., 2017). The plant materials indigenous to the K-soil or imported from RPS probably contributed to the increase in this genus, because a high abundance of Verrucomicrobia has been observed in the plant rhizosphere (da C. Jesus et al., 2010) and plant polymers or sugar were found to be necessary for growth of a representative member of *Spartobacteria* (Wolińska et al., 2017).

The taxa *Rhodanobacter* and *Ktedonobacterales* were present only in the K-soil and were reduced when amendments were added. *Saccharibacteria* genera *incertae sedis* also decreased after treatment with amendments. The presence of taxa such as *Nocardia*, *Spartobacteria* genera *incertae sedis*, *Noviherbaspirillum*, and *Pseudomonas* in K-soil contributed to the distance of the position of the K-soil control sample from the other samples. Some representatives of the genus *Niastella* have been reported to degrade chitin and cellulose (Chung et al., 2012; Weon et al., 2006), which might be a reason for the increase of *Niastella* in the RPS samples in K- and B-soils. *Pirellula* increased greatly in the biochar-treated K-soil samples, and less in the RPS sample in K-soil and all amendment samples in B-soil. This genus may be an important one, with a specific role in the biochar-amended soils. A subgroup of *Pirellula* has been found to be dominant in soils with compost treatments (Buckley et al., 2006).

Interestingly, the genus *Nitrosospira* decreased after all treatments, despite being present at significant levels in the K- and B-soil controls. This genus is well known as containing ammonia-oxidizing bacteria, and this autotrophic ammonia-oxidizing activity was highly inhibited with the addition of amendments, probably due to the supplementation of carbon sources (Erhunmwunse et al., 2019). The genus *Cystobacter*, a member of myxobacteria, seems to be related to the biochar amendments. They have a role in circulating organic substance cycles, as myxobacteria produce various extracellular enzymes to utilize insoluble organic substances (Dawid, 2000).

Sphingomonas, *Arthrobacter*, and *Massilia* showed the highest abundances in the K-soil amended with RPS_N. *Bacillus*, *Massilia*, and *Arthrobacter* had the highest abundances in the RPS-treated soil among the B-soils. Soil amendments often increased the absolute abundance of the genera *Arthrobacter*, *Massilia*, *Sphingomonas*, and *Bacillus*. All four of these genera perform specific functions in soils to increase plant growth and to maintain biogeochemical cycles. *Massilia* is a plant growth-promoting bacterial genus which has often been isolated in the plant rhizosphere. The production of indole acetic acid and siderophores, and antagonism to pathogens are some vital functions of *Massilia* in soil (Ofek et al., 2012). *Sphingomonas* could increase the adsorption of ions, synthesize siderophores to enhance iron uptake, and increase seed germination. Hence, *Sphingomonas* is considered as a plant growth-promoting bacterial genus (Yang et al., 2014). *Bacillus* is also a plant growth-promoting bacterial genus which is commonly found in soils. Mineral phosphate solubilization and production of organic acids such as lactic, acetic, isovaleric, and isobutyric acids have been found to be the most significant effects of *Bacillus* in soils (Hayat et al., 2010). The restoration of favorable growth conditions via addition of soil

amendments, such as reduction of metal stress, addition of growth surfaces, and supplementation of readily available carbon sources, might have increased *Massilia*, *Sphingomonas*, and *Bacillus* in K-soil. *Arthrobacter* species can utilize a wide range of organic substances as energy sources, and they can survive long periods under stress conditions, including a lack of food and high metal(loid) levels (Eschbach et al., 2003; Mongodin et al., 2006). In the K-soil, *Arthrobacter* had a similar absolute abundance in the control and in the RPS_N and RPS_C treatments, but was significantly increased in the RPS treatment. Hence, the addition of readily available carbon by RPS might have increased *Arthrobacter* in RPS more than in RPS_N and RPS_C.

4. Conclusion

The addition of the amendments RPS, RPS_N, and RPS_C resulted in convergence of the bacterial communities in each treatment. The K-soil bacterial community initially had a low diversity, due to the high Pb concentration combined with an acidic pH. This soil showed the strongest responses on treatments with amendments. The neutralization of pH, the reduction in Pb or Cd concentrations, and the supplementation of available carbon and minerals, and surface area could be possible reasons why the amendments affected soil microbial communities. Changes in bacterial communities were mirrored by the fatty acid profiles (Supplementary materials). The bacterial community structures at the phylum and genus levels suggested that the amendments might restore the normal bacterial community of the soils. The most abundant bacteria phyla of forest soils were observed in the studied soil and their abundance increased by the application of amendments. The addition of biochar resulted in the formation of a similar group of bacterial communities. This may reflect the similarity in the chemical composition of the added biochars. It was hypothesized that they could provide stabilizing effects on the soil bacterial community, returning it to its normal (non-contaminated) state. Further studies should be performed to gather additional evidence to support the findings of this study.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envint.2019.02.061.

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